

Somatic stem cell differentiation is regulated by PI3K/Tor signaling in response to local cues

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Summary statement

When stem cells move away from their niche, they must increase PI3K/Tor signaling in order to differentiate.

Abstract

Stem cells reside in niches that provide signals to maintain self-renewal, and differentiation is viewed as a passive process that depends on losing access to these signals. Here we demonstrate that differentiation of somatic cyst stem cells (CySCs) in the *Drosophila* testis is actively promoted by PI3K/Tor signaling, as CySCs lacking PI3K/Tor activity cannot properly differentiate. We find that an insulin peptide produced by somatic cells immediately outside of the stem cell niche acts locally to promote somatic differentiation through Insulin receptor (InR) activation. These results indicate that there is a local “differentiation” niche which upregulates PI3K/Tor signaling in the early daughters of CySCs. Finally, we demonstrate that CySCs secrete the Dilp-binding protein ImpL2, the *Drosophila* homolog of IGFBP7, into the stem cell niche, which blocks InR activation in CySCs. Thus, we show that somatic cell differentiation is controlled by PI3K/Tor signaling downstream of InR and that local production of positive and negative InR signals regulate the differentiation niche. These results support a model in which leaving the stem cell niche and initiating differentiation is actively induced by signaling.

Introduction

Stem cells maintain tissue homeostasis in adult organisms by balancing self-renewal and differentiation. Adult stem cells reside in niches that provide signals and space for self-renewal to occur (Losick et al., 2011). Thus, excess self-renewal is prevented by controlling the amount of self-renewal signals produced by the niche, as well as by limiting the available space at the niche. This results in a competition among stem cells to remain in the niche and access the self-renewal signals it provides (Klein and Simons, 2011). Much work has focused on the signals and the autonomous requirements for stem cell self-renewal. By contrast, little is known about how the rate of differentiation is regulated.

An underlying assumption in many studies is that differentiation is a “default” fate that stem cells adopt when deprived of maintenance cues or space at the niche. This view is informed by studies of germline stem cell (GSC) differentiation in the *Drosophila* ovary in which the stem cell self-renewal factor Dpp is required to repress transcription of the differentiation gene *bag of marbles* (*bam*) (Losick et al., 2011). Loss of this signal leads to de-repression of *bam* and subsequent differentiation, leading to the idea that differentiation needs to be actively repressed in the stem cells but happens by default once repression is lost. An alternative view is suggested by work in embryonic stem (ES) cells where self-renewal can be maintained by removing differentiation-inducing signals (Ying et al., 2008). However, ES cells represent a transitory and singular state of development that is distinct from adult stem cells where signaling from the niche maintains self-renewal in the long term. This model is supported by recent work in the *Drosophila* ovary, which suggests that somatic support cells called escort cells act as a differentiation niche to promote the timely progression of germ cells through differentiation

(Kirilly et al., 2011; Luo et al., 2015; Upadhyay et al., 2016; Wang et al., 2015).

We study differentiation in the *Drosophila* testis stem cell niche, a tissue that shares many characteristics with the ovary (Losick et al., 2011). In the testis, a physical niche called the hub supports two stem cell populations, GSCs and somatic cyst stem cells (CySCs). GSCs divide with oriented mitosis to give rise to gonialblasts that ultimately differentiate into spermatids. The CySCs divide to produce post-mitotic cyst cells. Each gonialblast is ensheathed by two cyst cells that are essential for the proper progression of the germline to meiosis (Fabrizio et al., 2003; Fairchild et al., 2015; Hardy et al., 1979; Kiger et al., 2000; Schulz et al., 2002; Shields et al., 2014; Tran et al., 2000). CySCs require JAK/STAT signaling for self-renewal, and the hub produces the JAK/STAT pathway ligand Unpaired (Upd) to maintain CySCs (Kiger et al., 2001; Leatherman and Dinardo, 2008; Tulina and Matunis, 2001). Additionally, CySCs require Hedgehog, Hippo, Slit/Robo and MAPK signals in order to remain at the niche and compete for space (Amoyel et al., 2016; Amoyel et al., 2013; Amoyel et al., 2014; Issigonis et al., 2009; Michel et al., 2012; Stine et al., 2014). In addition to intercellular signaling, many autonomous factors maintain CySCs, particularly the transcription factor Zfh1, which marks the CySC population (Leatherman and Dinardo, 2008). During cyst cell differentiation, Zfh1 expression is lost, while the differentiation marker Eyes Absent (Eya) is induced (Fabrizio et al., 2003; Leatherman and Dinardo, 2008). While it is not known whether cyst cell differentiation is a regulated process, it is thought to occur by default in cells that are displaced from the niche and can no longer receive self-renewal signals.

We previously showed that CySC clones in which the PI3K/Tor pathway is hyper-activated differentiate rapidly, leading to loss of these mutant stem cells from the niche (Amoyel

et al., 2014). However it was not known whether this reflected a requirement for PI3K/Tor activity during differentiation. The PI3K/Tor pathway is a major regulator of cellular growth, conserved across evolution (Grewal, 2009; Laplante and Sabatini, 2012; Loewith and Hall, 2011). PI3K is activated by receptor tyrosine kinases and phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP₂) lipids to create phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) (Fig. 1A). PIP₃ activates the kinase Akt1 leading to increased cellular growth through multiple effectors. One major effector and a separate growth regulator in its own right is Tor; Akt1 inactivates the Tor inhibitor Tsc1/2. Tor in turn acts in two major complexes (called Tor complex 1 (TORC1) and TORC2) to regulate multiple targets that affect all aspects of cellular metabolism. TORC1 and TORC2 are distinguished by different complex subunits and differential sensitivity to the inhibitor Rapamycin (Laplante and Sabatini, 2012; Loewith and Hall, 2011). Here we explore the physiological requirement for PI3K/Tor signaling in CySC differentiation and identify this pathway as a critical mediator of differentiation in stem cells.

Results

PI3K and Tor activity are upregulated during somatic differentiation

To determine whether PI3K and Tor are active during cyst cell differentiation, we took advantage of a reporter of PIP₃ levels that reflects PI3K activity (Fig. 1B and (Britton et al., 2002)) and an antibody against the phosphorylated form of eIF4-Binding Protein (p4E-BP), a direct target of Tor phosphorylation (Gingras et al., 1999; Miron et al., 2003). We labeled CySCs with Zfh1, a transcription factor that is expressed in approximately 40 cells, including the ~13 CySCs, that contact the hub, and their daughters that initiate differentiation (Fig. 1D", arrow and arrowhead, respectively) (Amoyel et al., 2014; Leatherman and Dinardo, 2008). CySCs are

identified as the first row of labeled nuclei around the hub (Fig. 1D-D'', arrow), while differentiating daughters are further away and express Eya (Fig. 1D''', arrowhead). Traffic jam (Tj), a large Maf protein, labels the nuclei of CySCs and early differentiating cells (Li et al., 2003). The PIP₃ reporter consists of GFP fused to the pleckstrin homology (PH) domain of Grp1, which binds PIP₃ (Britton et al., 2002). This GFP fusion is cytoplasmic, but when PIP₃ levels are high, it translocates to the plasma membrane (Fig. 1B). In control animals, we found that PH-GFP was predominantly cytoplasmic in CySCs surrounding the hub (Fig. 1C', arrow). However, we observed membrane-associated GFP in the cells immediately distal to the CySCs (Fig. 1C', arrowheads), suggesting that differentiating cyst cells have higher levels of PIP₃ and therefore higher PI3K activity, than CySCs. We confirmed that this reporter was indeed responsive to PI3K activity in the testis by over-expressing the activating subunit of PI3K, Dp110, or an RNAi against the PIP₃ phosphatase Pten in somatic cells. In these testes, we observed membrane localization of PH-GFP in all somatic cells, even CySCs (Fig. S1B',C' arrow). Similarly, we show that the Tor activity reporter p4E-BP is detected at low levels in the Zfh1-positive cells immediately closest to the niche (i.e., in CySCs) but is present at high levels in Zfh1-positive cells that are located two cell diameters away from the niche (Fig 1D-D'', arrow and arrowhead, respectively). This upregulation of p4E-BP is not maintained in mature cyst cells. Thus, high PI3K and Tor activity are detected in somatic cells immediately adjacent to the stem cells.

PI3K and Tor activity are required autonomously for cyst cell differentiation

As PI3K/Tor activity is observed early during cyst cell differentiation, we tested whether PI3K/Tor activity was required for CySCs to differentiate. We generated somatic clones mutant for the *Drosophila* PI3K *pi3k92E* (also called *Dp110*) using a null allele *Dp110^A* and for *Akt1*

using a null allele *Akt1*^q. As these clones are generated by mitotic recombination, clones are only generated upon CySC division. Control CySC clones with membrane-targeted GFP were recovered 7 days post clone induction (dpci) and could be identified as stem cells as they extended membrane processes that contacted the hub (Fig. 2A', arrow). These control clones also gave rise to differentiating offspring that move away from the niche and ensheathed spermatogonia (n=30 clones in 75 testes examined, Fig. 2A', arrowheads). Like control clones, *Akt1* or *Dp110* mutant CySCs that expressed Zfh1 and were close to the niche were recovered at 7 dpci (Fig. 2B',C',D',E', arrows). However, unlike control clones, we could not find *Akt1* or *Dp110* mutant cyst cells that ensheathed differentiating germ cells (n=12 *Akt1* clones in 64 testes examined and n=8 *Dp110* clones in 63 testes examined, Fig. 2B',D'), and no *Akt1* or *Dp110* mutant cells were positive for the differentiation marker Eya (Fig. 2C''',E'''), suggesting that proper somatic cell differentiation was blocked.

We next examined whether CySCs mutant for *Tor* exhibited a similar phenotype. We generated control or *Tor*^{AP} null MARCM clones that expressed nuclear GFP and analyzed these testes at 7 dpci. In testes with control clones, we observed labeled CySCs adjacent to the hub and differentiated cyst cells that have moved away from the hub (Fig. 3A,A', arrow and arrowhead, respectively). In contrast, *Tor* mutant clones were only found adjacent to the hub at 7 dpci (Fig. 3B,B', arrows, quantified in 3C). We labeled testes carrying marked control or *Tor*^{AP} mutant clones with the stem cell marker Zfh1 and the differentiation marker Eya to determine whether *Tor* mutant cells become mature cyst cells. At 7 dpci, we always observed control clones composed of both Zfh1-expressing CySCs and Eya-expressing cyst cells (n=15 clones, Fig. 3E', arrow and arrowhead, respectively). In contrast, all *Tor*^{AP} mutant clones examined contained only cells expressing Zfh1 and no cells positive for Eya (n=8 clones, P<0.0001 Fisher's exact

test compared to control clones, Fig. 3F', arrow), similar to results we observed for *Akt1* and *Dp110* mutant clones labeled with nuclear GFP (Fig. 2C,E). To determine whether *Tor* mutant cyst cells were differentiating but were not subsequently recovered because they were dying, we prevented apoptosis within the clones by expressing the baculovirus caspase inhibitor P35 (Fig. 3D). These clones were recovered at higher rates than *Tor* mutant clones alone (45% vs 14%, n=38 and 131 testes, respectively), indicating that *Tor* mutant cells are eventually lost by apoptosis and that co-expressing P35 can block their death. However, these clones still did not contain any differentiated cyst cells (Fig. 3D', arrows). Additionally, as *Tor* and *Akt1* null mutant CySCs had an apparent proliferation defect, we tested whether CySCs mutant for an *Akt1* (*Akt1*³) or a *Tor* hypomorphic allele (*Tor*^{A948V}) could differentiate. These mutant CySCs proliferated better, generating more labeled cells within the clone, which could persist up to 7 or 14 days without dying but did not differentiate (Fig. S2A, compare with Fig. 2D; Fig. S2B,C compare with Fig. 3B). Thus, our data suggest that PI3K/Tor activity is autonomously required for CySCs to differentiate into cyst cells, independently of proliferation or survival. We note that CySC clones with a proliferation defect can differentiate, as was shown for CySCs clones lacking *string* (*stg*), the *Drosophila* Cdc25 homolog, and in fact CySCs with impaired proliferation cannot be maintained in the niche (Inaba et al., 2011). Therefore, a proliferation defect cannot account for the lack of differentiation of *Tor* mutant clones.

PI3K acts through TORC1 to regulate differentiation of somatic cells

Next we asked whether decreased PI3K/Tor in the entire somatic lineage affected its progression. We knocked down the PI3K effector, *Akt1*, to determine whether the PI3K pathway is required for normal differentiation. In control testes, *Zfh1* is expressed in CySCs and their

immediate offspring, concentrated around the hub (n=46 testes, Fig. 4A,A'). As they differentiate, cyst cells lose *Zfh1* expression and begin to express *Eya* (Fig. 4A'', arrowhead). In testes with somatic depletion of *Akt1*, we observed somatic cells expressing *Zfh1* located many cell diameters from the hub (n=21/21 testes examined, Fig. 4B,B'). Moreover, we detected no or low levels expression of *Eya* in these samples (Fig. 4B''').

We obtained a similar phenotype of impaired somatic differentiation when we knocked down *Tor* in somatic cells (n=15/16 testes examined, Fig. 4C). *Tor* acts in two complexes, called TORC1 and TORC2. These complexes are distinguished by different subunits, although *Tor* itself is common to both (Laplane and Sabatini, 2012). Moreover, the two *Tor* complexes differ in their sensitivity to Rapamycin (Jacinto et al., 2004). We tested which *Tor* complex was required in cyst cell differentiation by knocking down the TORC1 complex member Raptor and observed a similar phenotype to *Tor* knockdown alone (n=17/17 testes examined, Fig. 4D). Consistent with this observation, testes from adult males fed the TORC1 inhibitor Rapamycin closely resembled testes in which *Tor* was depleted from the somatic lineage (n=18/18 testes examined, Fig. 4E). We observed ectopic *Zfh1*-expressing cells away from the hub and a lack of *Eya*-positive cells (Fig. 4E',E''', respectively). Conversely, somatic depletion of Rictor, a TORC2-specific component, did not impact cyst cell differentiation as *Eya*-positive cyst cells were readily observed (n=13/13 testes appeared similar to control, Fig. S3). These results indicate that *Tor* acts in the TORC1 complex to promote cyst cell differentiation. Consistent with this, germ cells from testes somatically depleted for *Tor* or from testes of Rapamycin fed males were maintained in an early, undifferentiated state (Fig. S4), presumably due to the role of CySCs in maintaining GSCs or to the requirement of mature cyst cells in promoting germline differentiation (Kiger et al., 2000; Leatherman and Dinardo, 2010; Schulz et al., 2002; Tran et

al., 2000).

We ruled out the possibility that the maintenance of Zfh1 expression in somatic cells depleted for Tor signaling resulted from ectopic activation of Stat92E, a key self-renewal pathway in CySCs. Stabilized Stat92E is a well-established marker of its activation (Flaherty et al., 2010). In wild type testes, stabilized Stat92E is observed only in GSCs and CySCs in contact with the niche (Fig. S5A), and this pattern is not perturbed in somatic cells depleted for Tor (Fig. S5B).

Finally, we tested whether PI3K activity was linearly upstream of Tor activity in somatic cells of the testis. In testes in which Akt1 was knocked down in somatic cells, we observed a marked decrease in p4E-BP levels, indicating that Tor activity is downstream of PI3K in these cells (Fig. S6B). Moreover, we rescued the defective differentiation of cells with reduced Akt1 by concomitantly knocking down the Tor inhibitor Tsc1 (Fig. 4F). Although Akt1 knock down alone prevented cyst cell differentiation and blocked Eya expression (n=1/32 testes contained Eya-positive cyst cells, Fig. 4B), Tsc1 knock down together with Akt1 RNAi led to robust Eya expression and apparently normal progression of both cyst cells and germ cells through differentiation (n=22/23 testes contained Eya-positive cyst cells, Fig. 4F). These results demonstrate that PI3K signaling through TORC1 is essential for differentiation of the cyst lineage in the *Drosophila* testis.

Somatic cyst cells lacking PI3K and Tor activity remain proliferative

CySCs are the only dividing somatic cells in wild type testes, and differentiating somatic cells exit the cell cycle and become quiescent. Thus, the ability to divide is a marker of the CySC state. Although CySCs lacking PI3K/Tor proliferated poorly, we showed that clones of

hypomorphic mutations in *Akt1* and *Tor* do expand but yet do not differentiate (Fig. 2 and S2). Therefore, we asked whether CySC fate was maintained in lineage-wide knock downs and assessed proliferative potential as a marker of the CySC state. We examined the S-phase marker 5-ethynyl-2'-deoxyuridine (EdU) in control testes compared to Tor and Raptor knock down testes. In controls, only somatic cells near the hub incorporated EdU (n=0/34 testes contained at EdU-positive cyst cells located away from the hub, Fig. 5A-A'', arrows), while germ cells away from the hub replicated synchronously in 2, 4, 8 or 16-cell cysts. In testes with somatic depletion of Tor or the TORC1 component Raptor, we observed EdU-positive somatic stem cells next to the niche (Fig. 5B-C'', arrows). Importantly, we also detected EdU-positive somatic cells located many cell diameters away from the hub, suggesting that these cells maintained a stem cell-like fate (n=10/17 and 10/16 testes contained EdU-positive cyst cells located away from the hub, for Tor and Raptor knockdown, respectively, Fig. 5B-C'', arrowheads). Similarly, EdU-positive somatic cells located many cell diameters from the hub were observed when we knocked down Akt1 in somatic cells (data not shown). These results indicate that somatic cells lacking PI3K/Tor can maintain stem-like features.

Signaling controls the localization of differentiation

Our results indicate that differentiation is induced by active signaling, suggesting that, as with stem cell self-renewal, there is a “niche” for stem cell differentiation (i.e., an environment that promotes a certain cell fate, in this case differentiation) (Kirilly et al., 2011). We sought to identify such a niche by determining how the pattern of p4E-BP is established. Indeed, it is striking that p4E-BP is low in CySCs, but high in the cells immediately adjacent to them, suggesting that the spatial patterning of Tor activity is highly regulated. Since PI3K is upstream

of Tor activity (Fig. 4F, S6B), activation of PI3K is limiting in this context. Removing InR in all somatic cells mimicked PI3K pathway loss of function (Fig. S7A compare to control in Fig. 4A), which supports the model that PI3K is downstream of the sole *Drosophila* Insulin Receptor (InR) (Grewal, 2009). Similar to *Dp110* and *Akt1* clones, CySC clones mutant for *InR* were unable to differentiate and remained adjacent to the niche without encysting germ cell cysts (Fig. S7B, arrows, compare to control in Fig. 2A). Moreover, adult males carrying hypomorphic mutations in *InR* have strongly reduced fertility (Tatar et al., 2001; Ueishi et al., 2009). Therefore, a *Drosophila* insulin-like peptide (Dilp) likely signals to induce CySC differentiation.

The *Drosophila* genome encodes seven Dilps, but one of these, Dilp6, is functionally and molecularly homologous to Insulin-like Growth Factor (IGF), which acts to promote tissue growth during development, and is therefore a good candidate to be a local inducer of PI3K activity (Brogiolo et al., 2001; Okamoto et al., 2009; Slaidina et al., 2009). We observed that *dilp6-gal4* was expressed in differentiating cyst cells (Fig. 6A) and overlapped partly with the domain of p4E-BP expression (Fig. 6B-B'', arrowhead), suggesting it may act as a juxtacrine or paracrine factor. *dilp6* mutants displayed a modest but significant accumulation in the number of Zfh1-positive, Eya-negative cells compared to controls (51.9 for *dilp6*⁴¹ and 51.4 for *dilp6*⁶⁸, compared to 40.1 for control, P<0.0001 and P<0.0001 Dunn's multiple comparison test, n=27, 36 and 20, respectively). This is due to autonomous action within the cyst cell lineage, as knockdown of Dilp6 with Tj-Gal4 led to the same phenotype (Fig. 6D, 51.9 Zfh1-positive Eya-negative cells in Tj>*dilp6* RNAi compared to 45 cells in Tj>+, P=0.0007, Mann-Whitney test, n=20 and 19, respectively). Moreover, p4E-BP staining was reduced but not absent in *dilp6* mutants (Fig. 6E',F', brackets), suggesting that Dilp6 acts partially redundantly with other Dilps to promote differentiation in the testis. Since no other Dilps are expressed near the niche (Wang

et al., 2011), it is likely that systemic Dilps are also involved in activating PI3K during CySC differentiation. Indeed, loss of brain-derived Dilps causes defects in germ cell differentiation (Ueishi et al., 2009) and systemic Dilps can affect GSC behavior in both male and female gonads (LaFever and Drummond-Barbosa, 2005; McLeod et al., 2010), indicating that systemic signals reach the gonadal stem cell niches. In turn, if systemic signaling activates PI3K, the pattern of PI3K activation implies that signaling is inhibited in CySCs.

ImpL2 inhibits PI3K activity in CySCs to spatially separate self-renewal and differentiation.

To explore this possibility, we examined the expression and role of *Imaginal morphogenesis protein-Late 2 (ImpL2)*, the *Drosophila* homolog of IGFBP7, a secreted antagonist of Dilp signaling (Alic et al., 2011; Arquier et al., 2008; Honegger et al., 2008). ImpL2 was upregulated by JAK/STAT signaling and was previously shown to be expressed in cells adjacent to the hub (Terry et al., 2006), suggestive of a role in repressing Dilp activity near the hub. We confirmed using an enhancer trap that *ImpL2* expression was detected specifically in CySCs around the hub (Fig. 7A', arrows) and an antibody against ImpL2 revealed punctate staining around the hub (Fig. S8A). This staining was lost when ImpL2 was knocked down using Tj-Gal4 and increased when ImpL2 was mis-expressed (Fig. S8B,C), indicating that ImpL2 is produced by the somatic lineage.

Since ImpL2 has been identified as a secreted Dilp antagonist, we tested whether ImpL2 was repressing PI3K activity in CySCs by analyzing p4E-BP expression as a readout for PI3K/Tor pathway activity in these cells. In testes in which ImpL2 was knocked down, we observed many p4E-BP-positive cells in contact with the hub (Fig. 7C', arrow, 4.4 p4E-BP-

positive cells contacting the hub, compared to 1.6 in control testes, $P < 0.0001$, Mann-Whitney test, $n = 22$ for $Tj > ImpL2$ RNAi and $n = 15$ for $Tj > +$ control). Conversely, over-expressing *ImpL2* led to excess *Zfh1*-positive cells and a delayed onset of *Eya* expression, indicating that CySC differentiation was hindered ($n = 23/25$ testes examined, Fig. 7D). These results are consistent with *ImpL2* blocking InR activation in somatic cells and preventing their differentiation by inhibiting PI3K/Tor pathway activity. Together, our results suggest that both activating and inhibitory ligands localize the differentiation niche and spatially separate differentiation from self-renewal.

Discussion

We show that PI3K/Tor activity is required for the differentiation of somatic stem cells in the *Drosophila* testis. Additionally, we identify a “differentiation” niche immediately adjacent to the stem cell niche that, through the local production of Dilps, leads to the upregulation of PI3K/Tor activity in early CySC daughters and to their commitment to differentiation. The secretion of *ImpL2* by CySCs antagonizes the initiation of differentiation in CySCs by blocking available Dilps in the stem cell niche. As a result, CySCs receive little free Dilp ligands. However, as their daughters move away from the hub, they encounter increasing levels of Dilps and decreasing levels of *ImpL2*, which leads to the upregulation of PI3K/Tor signaling and proper somatic cell differentiation. The fact that *ImpL2* is upregulated by the main self-renewal signal (i.e., JAK/STAT (Terry et al., 2006)) in CySCs leads to a model accounting for the spatial separation of the stem cell niche and the differentiation niche.

A “differentiation” niche in the testis

Our results are consistent with a model in which autocrine or paracrine production of Dilp6 by early cyst cells serves as a differentiation niche in the testis, defining where in the tissue upregulation PI3K/Tor signaling - a prerequisite for differentiation - occurs. This differentiation niche is critical for somatic development because stem cell markers like Zfh1 are maintained in the absence of signals like PI3K/Tor. Notably, JAK/STAT activity is not expanded outside of the niche upon somatic loss of PI3K/Tor signaling, suggesting that differentiation signals play a critical role in downregulating stem cell factors. Intriguingly, recent studies in the *Drosophila* ovary have identified a differentiation niche in this tissue: autocrine Wnt ligands produced by somatic support escort cells regulate escort cell function, proliferation and viability (Luo et al., 2015; Upadhyay et al., 2016; Wang et al., 2015). Taken together, these studies reveal that at least in *Drosophila* gonads, there is a defined region immediate adjacent to the stem cell niche where autocrine production of secreted factors induces the differentiation of somatic cells, which in turn promote development of the germ line.

Role of insulin in stem cell niches

Several studies have examined the role of insulin signaling in gonadal stem cells. In both testes and ovaries, systemic Dilps have been shown to affect stem cell behavior (Hsu and Drummond-Barbosa, 2009; Hsu et al., 2008; LaFever and Drummond-Barbosa, 2005; McLeod et al., 2010; Ueishi et al., 2009). In both tissues, nutrition through regulation of systemic insulin controls the proliferation rate of GSCs (Hsu et al., 2008; McLeod et al., 2010). Our data showing that *Akt1*, *Dp110* or *Tor* mutant CySC clones proliferate poorly are consistent with these findings and indicate that basal levels of insulin signaling are required for the proliferation and/or survival of both stem cell pools in the testis. Our work also demonstrates that production of a secreted

Insulin binding protein ImpL2 by CySCs reduces available Dilps in the stem cell niche, and ImpL2 in the niche milieu should reduce insulin signaling in GSCs and CySCs. While these data seemingly contradict the results that insulin is required for GSC maintenance (McLeod et al., 2010), we suggest a model in which low constitutive levels of insulin signaling are required for stem cell proliferation and that higher levels are required to induce stem cell differentiation.

PI3K/Tor signaling and differentiation of stem cells

Prior reports have found that both male and female flies with reduced Insulin or Tor activity are sterile (Tatar et al., 2001; Ueishi et al., 2009; Zhang et al., 2006), and the results presented here suggest that this is due at least in part to a lack of somatic cell differentiation. Our results indicate that Dilp6, the IGF homolog, plays a local role in CySC differentiation, but acts redundantly with other presumably systemic factors, suggesting that both constitutive and nutrient-responsive inputs control CySC differentiation. Indeed, we show that in addition to controlling the proliferation of stem cells, systemic insulin is required for their differentiation, as the poorly proliferative *Akt1*, *Dp110* or *Tor* mutant CySC clones do not differentiate and eventually die by apoptosis. This combination of reduced proliferation and increased apoptosis may explain why other studies suggest that Tor is required for self-renewal in GSCs; indeed prior reports indicate that while *Tor* mutant GSCs are lost, hyper-activation of Tor leads to faster loss of GSCs through differentiation (LaFever et al., 2010; Sun et al., 2010) and recent work indicates that lineage-wide Tor loss blocks the differentiation of GSCs (Sanchez et al., 2016). Our use of hypomorphic alleles enabled a genetic separation of the proliferative effects and differentiation requirements of PI3K and Tor in CySCs. Finally, there is evidence that PI3K/Tor activity promotes differentiation of stem cells in gonads in mammals, suggesting that our

findings may reflect a conserved role of Tor activity in promoting germ cell differentiation, both through autonomous and non-autonomous mechanisms involving somatic support cells (Adhikari et al., 2010; Busada et al., 2015; Hobbs et al., 2010; Reddy et al., 2008).

Moreover, it seems likely that Tor activity may be a more general requirement for the differentiation of many stem cell types, as increased PI3K or Tor has been shown to induce differentiation in many instances. In particular, mouse long term hematopoietic stem cells are lost to differentiation when the PI3K inhibitor *Pten* is mutated (Signer et al., 2014; Yilmaz et al., 2006), while *Drosophila* intestinal stem cells differentiate when Tor is hyperactive due to Tsc1/2 complex inactivation (Kapuria et al., 2012; Quan et al., 2013). Moreover, inhibition of Tor activity by Rapamycin promotes cellular reprogramming to pluripotency, while cells with increased Tor activity cannot be reprogrammed (Chen et al., 2011; He et al., 2012), suggesting a conserved role for Tor signaling in promoting differentiated states.

Materials and Methods

Fly stocks and husbandry

We used the following fly stocks: Oregon R; *Tor*^{AP}, *FRT*^{40A}; *Tor*^{A948V11}, *FRT*^{40A}; *FRT*^{82B}, *Akt1*^q; *FRT*^{82B}, *Akt1*³; *FRT*^{82B}, *InR*^{E19}; *FRT*^{82B}, *Dp110*^A; Tj-Gal4; UAS-Akt1 RNAi (TRiP HMS00007); UAS-Tor RNAi (TRiP GL00156); UAS-Tsc1 RNAi (TRiP HMJ21477); UAS-Raptor RNAi (TRiP HMS00124); UAS-Pten RNAi (TRiP JF01859); UAS-Dp110; UAS-P35; UAS-Impl2 (gift of E. Hafen); UAS-Impl2-RNAi (VDRC #30931); UAS-Dilp6 RNAi (TRiP JF01348); Dilp6-Gal4 (NP1079-GAL4, DGRC Stock Center, Kyoto); Impl2-GFP (Impl2^{MI01638-GFSTF.0}) (Nagarkar-Jaiswal et al., 2015); tGPH (PH-GFP) (Britton et al., 2002). Tj-Gal4 is expressed in CySCs and in early differentiating cyst cells. To determine the Zfh1-expressing population in the testis, we counted all Zfh1-positive and Eya-negative cells in an entire confocal Z stack.

Positively marked clones were generated by the MARCM technique after a single 1 hour heat-shock at 37°C (Lee and Luo, 1999). Lineage-wide mis-expression or depletion was achieved using the Gal4/UAS system (Brand and Perrimon, 1993). A *gal80^{ts}* transgene was used when depleting Akt1 (McGuire et al., 2004).

Rapamycin was obtained from LC Laboratories, Woburn, MA. Rapamycin-containing fly food was prepared by adding 100µL of a 4mM stock solution in ethanol to each food vial and letting it air dry. Flies were transferred to fresh Rapamycin-containing food every two days. Flies were raised at 25°C except Tj-Gal4 crosses which were raised at 18°C and adult flies of the correct genotype were maintained at 29°C for 10 days to achieve maximum Gal4 activity.

Immunohistochemistry

The following primary antibodies were used : rabbit anti-Zfh1 (gift of R. Lehmann), 1:5000;

guinea pig anti-Zfh1 (gift of J. Skeath), 1:500; guinea pig anti-Traffic Jam (gift of D. Godt), 1:3000; rabbit anti-Phospho-4E-BP1 (Thr37/46, Cell Signaling), 1:100; goat anti-Vasa (Santa Cruz), 1:100; mouse anti-Fas3 (DSHB), 1:20; mouse anti-Eya (DSHB), 1:20; mouse anti-Dlg (DSHB), 1:20; rat anti-Impl2 (gift of J. Natzle), 1:500; rabbit anti-GFP (Life Technologies), 1:500; chicken anti-GFP (Immunology Consultants), 1:500.

EdU incorporation and detection were carried out as previously described (Amoyel et al., 2014).

Statistics

Non-parametric data are subjected to two-tailed Mann-Whitney tests and Dunn's multiple comparison tests. Chi squared was determined by Fisher's exact test. Significance is determined and indicated. All data and graphs were analyzed in GraphPad Prism 6 software.

Competing interests

The authors declare no competing interests.

Author contributions

M.A. designed the experiments with contributions from E.A.B.; M.A., K.-H.H., S.R.M. and E.A.B. performed the experiments and analyzed the data; M.A. and E.A.B. wrote the manuscript.

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Figure legends

Figure 1. PI3K and Tor activity are observed during CySC differentiation. A. Simplified model of the PI3K/Tor pathway. Here PI3K is activated by the Insulin receptor (InR) tyrosine kinase following binding of a *Drosophila* Insulin-like peptide (Dilp). The InR phosphorylates PI3K, which in turn phosphorylates PIP₂ lipids to create PIP₃. Pten is a lipid phosphatase that catalyzes the reverse reaction. Subsequently, PIP₃ activates Akt, which inactivates the Tor inhibitor Tsc1/2. The TORC1 complex is then activated and leads to the phosphorylation of S6K and 4E-BP. The secreted Dilp antagonist ImpL2 blocks Dilps from binding to InR. Phosphorylation events are indicated by yellow stars. B. The PH-GFP reporter of PIP₃ consists of GFP fused to the pleckstrin homology (PH) domain of Grp1, which binds PIP₃. This GFP fusion is cytoplasmic, but when PIP₃ levels are high, it translocates to the plasma membrane. C. The PI3K reporter PH-GFP (green) was predominantly cytoplasmic in CySCs around the hub, indicating low PI3K activity. Arrow in C', C''' marks a CySC with low membrane PH-GFP. Membrane-associated PH-GFP was observed as cyst cells begin to differentiate, one further cell diameter away from the hub. Arrowheads in C', C''' mark differentiating cyst cells that have upregulated membrane PH-GFP. Tj (blue, C'') labels CySCs and early cyst cells, Dlg (red, C'') marks somatic cell membranes. D. The Tor activity reporter p4E-BP (green) was detected at high levels in somatic cells adjacent to stem cells. Arrow in D-D'' marks a Zfh1-positive CySC next to the niche that has low p4E-BP. Arrowhead in D-D'' labels a Zfh1-positive early CySC daughter cell primed for differentiation that is one cell diameter away from the CySCs and that has high levels of p4E-BP. Arrowhead in D''' marks an Eya-positive differentiating cyst cell. Fas3 (blue, D'') marks the hub, CySCs are the Zfh1-positive cells (red, D'') that are closest to the hub.

Figure 2. *Dp110* and *Akt1* are required autonomously for CySC differentiation. MARCM clones labeled by GFP expression. A,B,D. Clones marked with membrane-targeted GFP (green, A',B',D'). Vasa (red, A'',B'',D'') labels germ cells and Tj (blue, A''',B''',D''') marks somatic cells. At 7 dpci, control clones (A) contained labeled CySCs contacting the hub (A', arrow) and differentiated cyst cells ensheathing large germline cysts (A', arrowheads). By contrast, *Dp110* (B',B''', arrows) and *Akt1* (D',D''', arrow) mutant clones contained labeled CySCs contacting the hub but lacked labeled differentiated daughter cells. Note that in B only part of the hub is in the plane. C,E. *Dp110* (C) or *Akt1* (E) mutant clones marked with nuclear GFP (green, C',E', arrows). Mutant cells expressed the CySC marker Zfh1 (red, C'',E'', arrows) and lacked expression of the differentiation marker Eya (blue, C''',E'''). The hub is outlined by a red dotted line.

Figure 3: Tor is required autonomously for CySC differentiation. A. Control nuclear GFP-labeled clone at 7 dpci showing labeled CySCs adjacent to the hub (A', arrow) and differentiated cyst cells that have moved away from the hub (A', arrowhead). A' shows a maximum projection of the confocal stack, to display all the labeled GFP-positive cells in this sample. B. *Tor^{AP}* mutant clone at 7 dpci. All the mutant cells (B', arrows) in the clone remained close to the hub, as seen in the maximum projection in B'. C. Quantification of the distance between marked cells in clones and the hub. *Tor^{AP}* clones remained significantly closer to the hub than control clones ($P < 1 \times 10^{-4}$ Mann-Whitney). D. Preventing cell death in *Tor^{AP}* clones at 7 dpci by expressing the caspase inhibitor P35 does not lead to the recovery of differentiated cells (D', arrows). E. Control clones contained both Zfh1-expressing CySCs (E',E''', arrow) and Eya-expressing differentiated cyst cells (E',E'', arrowhead). F. By contrast, *Tor^{AP}* mutant clones contained only Zfh1-

expressing cells (F', F'', arrow) and no Eya-expressing cells (F''). The hub is outlined with a dotted line in all panels.

Figure 4. PI3K or TORC1 pathway knock down in the somatic lineage prevents CySC

differentiation. A. A Control (Tj>+) testis showing Zfh1 (green, A') in CySCs and early cyst cells near the hub and Eya (blue) in differentiated cyst cells (A'', arrowhead). Vasa (red, A'') labels the germ line. Germ cells grow larger as they differentiate and move away from the hub. B. Akt1 knock down in the somatic lineage led to expansion of the Zfh1-expression domain (green, B') away from the hub, loss of Eya expression (blue, B'') and prevented normal germ cell development, as only small germ cells were seen (red, B''). C. Tor knock down in somatic cells by RNAi. Many Zfh1-expressing cells (green, C') were seen far from the hub, while Eya expression was absent (blue, C''). Germ cell differentiation was also disrupted (C''). D. Knock down of the TORC1 component Raptor caused a similar phenotype to Akt1 and Tor knock down. Zfh1 expression (D') was maintained in cells distant to the hub, while Eya was absent (D'') and germ cell differentiation was blocked (D''). E. Feeding flies the TORC1 inhibitor Rapamycin prevented differentiation, as Eya expression was absent (E'') while Zfh1 (E') expanded and germ cells (E'') did not differentiate properly. F. Hyper-activating Tor by knock down of Tsc1 rescues the loss of differentiation observed when Akt1 is knocked down by RNAi. Note that in contrast to Akt1 knock down alone (B), concurrent knock down of Tsc1 leads to robust Eya expression (F'') and rescue of germ cell differentiation. The hub is outlined with a dotted line in all panels.

Figure 5: PI3K/Tor pathway knock down in the somatic lineage blocks quiescence. A. In a

control (Tj>+) testis, only CySCs near the hub underwent S-phase, revealed by EdU incorporation (A-A''', arrows). B,C. Like control testes, EdU incorporation was observed in CySCs next to the niche in testes with somatic knock down of Tor or Raptor (B-C''', arrows), but unlike control testes, EdU incorporation was also observed in somatic cells far from the hub (B-C''', arrowheads). Tj is green, EdU is red and Vasa is blue in A-C. The hub is outlined with a dotted line in all panels.

Figure 6. Localized Dilp6 expression promotes CySC differentiation. A. Dilp6-Gal4 driving expression of UAS-GFP in a control testis. GFP (green, A') is expressed in differentiating cyst cells. Vasa (red, A'') marks germ cells and Tj (blue, A''') labels somatic cells. B. *dilp6*>GFP (green, B') expression is partially overlapping with p4E-BP (red, B''), suggesting that Dilp6 could act in a juxtacrine or autocrine manner. Tj labels somatic cells in blue (B'''). Arrowhead in B-B''' indicates a Tj-positive somatic cell that expresses the Dilp6 driver and p4E-BP. C. A control (Tj>+) testis stained with Zfh1 (green, C'). D. Knockdown of Dilp6 in somatic cells results in an accumulation of Zfh1-expressing cells (green, D'). In C, D Vasa is red and Eya and Fas3 are blue. More Zfh1-positive cells are seen in a Tj>Dilp6 RNAi testis (D) than in a control (C). E,F. p4E-BP expression (green, E',F') in control (E) or *dilp6* mutant testes (F). Note the reduction both in the width of the p4E-BP domain and in the expression levels (brackets in E',F'). Tj labels somatic cells in red (E'',F'') and Fas3 marks the hub in blue (E''',F'''). The hub is outlined with a dotted line.

Figure 7. CySCs produce ImpL2 to antagonize local Dilp signaling in stem cells. A. ImpL2-GFP expression (green) is detected in CySCs contacting the hub (A',A''', arrows), in a pattern

complementary to p4E-BP expression (red, A''). Tj is blue (A'''). B,C. p4E-BP expression (green, B',C') is altered in testes with somatic depletion of ImpL2. Tj (red) marks somatic cells and Fas3 (blue) labels the hub. p4E-BP is detected in differentiating cyst cells, two cell diameters away from the hub in a control testis (B). However, high levels of p4E-BP are detected in CySCs immediately adjacent to the hub when ImpL2 is knocked down (C', arrow). D. Somatic over-expression of ImpL2 results in an accumulation of Zfh1-expressing cells (green, D') and a delay in the onset of Eya expression (blue, D'''). Vasa labels germ cells. In all panels, the hub is outlined with a dotted line.













